P2X$_7$ receptor-Pannexin1 complex: pharmacology and signaling

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The ionotropic purinergic P2X$_7$ receptors (P2X$_7$R) initially respond to agonists with the opening of a nonselective cation channel (24, 32). Prolonged exposure, however, leads to opening of a large pore that is permeable to molecules as large as 831 Da (44). Although this dual mode of function has been observed in many cell types, the pore has been reported to be absent in Xenopus oocytes in which P2X$_7$ cRNA is expressed (40), leading to the hypothesis that the small-conductance cation pathway may correspond to the P2X$_7$R (whose other family members display such permeation upon ATP binding: 32), whereas the pore might be formed by another molecule that is recruited in the response. A candidate molecule recently proposed for the highly permeable pore is pannexin 1 (Panx1) (30, 38). This molecule, found in chordates, displays a low but significant degree of homology to innexins, the proteins that form gap junction channels in invertebrates (5, 52). Although Panx1 overexpression in Xenopus oocytes has been reported to result in a low degree of functional gap junction formation (10), formation of such intercellular channels by Panx1 in mammalian cells is unlikely (16, 21) given the presence of a glycosylation site in the extracellular docking domain of this protein (7, 8, 37). Nevertheless, Panx1 readily forms nonjunctional channels in both Xenopus oocytes and in mammalian cells; these channels can be activated by stretch (3), elevations in intracellular Ca$^{2+}$ (28), and depolarization above about +20 mV (3, 9). Moreover, Panx1 channels can be activated by prolonged stimulation of P2X$_7$R (30, 38). In mammalian cells, activation of the P2X$_7$R-Panx1 complex has been implicated in the processing of the proinflammatory cytokine interleukin-1β and the activation of caspase-1 in immune cells (38, 39) and in the release of ATP from several cell types, including astrocytes (41, 46), erythrocytes (29), and taste bud cells (22).

It has been proposed that nonjunctional (hemi-) channels can be formed by the gap junction proteins, the connexins, and that these may perform the function of large-conductance, highly permeable pores, allowing release of ATP and/or glutamate from astrocytes (45, 51; reviewed in Ref. 43). The stimulus used to activate connexin hemichannels is removal of divalent cations, a treatment long known to enhance P2X$_7$R sensitivity to agonists (49). Moreover, evidence for such a role of connexin hemichannels is heavily dependent on use of compounds known to block intercellular gap junction channels, which have recently been shown to also block the P2X$_7$R-mediated uptake of dye molecules (46). It therefore remains possible that Panx1 through its association with P2X$_7$R may mediate the dye uptake and molecule release attributed to connexin hemichannels.

A previous study reported inhibition of flux of dye molecules only by one of several gap junction blockers tested and that dye uptake was not strictly correlated with large-conductance channel activation (38). Therefore, the present study was undertaken to further explore the pharmacological action of compounds known to block gap junctions on the cation channel and Panx1 pore associated with P2X$_7$R and to gain insight into mechanisms involved in the interaction between these two groups of proteins. Our results provide further support that Panx1 is the permeabilization pore activated in association with the P2X$_7$R and demonstrate that the antimalarial drug mefloquine is a highly potent inhibitor of Panx1 channels. We also provide, for the first time, evidence for the participation of a Src tyrosine kinase mediating the initial steps of the signal transduction pathway.
transduction pathway involved in the activation of Panx1 channels through the P2X-R.

**MATERIALS AND METHODS**

**Cell culture.** The mouse macrophage cell line J774 (obtained from Dr. A. Casadevall, Albert Einstein College of Medicine) was used in this cell. Cells were cultured in DMEM, supplemented with 10% FCS (GIBCO), and 1% of penicillin/streptomycin and maintained in an humidified chamber with 5% CO2 at 37°C.

Electrophysiology. J774 cells were plated on cover slips 24–48 h before recordings. The whole cell patch-clamp configuration was performed at room temperature on cells bathed in external solution containing (in mM): 147 NaCl, 10 HEPES, 13 glucose, 2 CaCl2, 1 MgCl2, and 5 KCl, pH 7.4. The pipette solution contained (in mM): 130 CsCl, 10 EGTA, 10 HEPES, and 0.5 CaCl2. Activation of Panx1 channel by voltage was performed using a ramp protocol by applying voltages from a holding potential of −60 mV to final values of +100 mV. To analyze the participation of Panx1 channels in agonist-induced P2X-R activation, cell membrane potential was held at −60 mV after forming high-resistance (1–10 GΩ) seals and the whole cell recording configuration. 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (BzATP, 50 µM) and ATP (1–5 mM) were superfused for 5 s. After the first response to the agonist, the gap junction channel blockers carbenoxolone (50 µM; CBX), mefloquine (10–100 nM; MFO), and flufenamic acid (100–300 µM; FFA) and the P2X-R antagonist KN-62 (1 µM) were superused for 5 min before the addition of 50 µM BzATP in the presence of the blockers. Cells were then washed with external solution for several minutes and further stimulated with 50 µM BzATP to evaluate reversibility of the blockers and the P2X-R antagonist. All data were recorded through an Axopatch 200B amplifier and digitized using an Axon Instruments Digitizer (Molecular Devices). Clampex 6.0 was used for recording and the Clampfit 9.0 program for analysis.

**Oocytes.** Preparation of oocytes and electrophysiological recording were performed using the two-microelectrode voltage-clamp technique, as previously described (30). Human Panx1 (MR51) was kindly provided by Dr. Graeme Bolger, University of Alabama. Panx1, in Bluescript, was linearized with Kpn I and human Panx1-(kindly provided by Dr. Annmarie Suprenant, University of Manchester, Manchester, United Kingdom), in pcDNA3, with Avr II. In vitro transcription was performed with the polymerases T3 (Panx1) and T7 (P2X-R) using the mMessage mMachine kit (Ambion, Austin, TX). In vitro transcribed mRNAs (~20 nl) were injected in Xenopus oocytes. The oocytes were incubated at 18°C for 18–24 h in oocyte Ringer solution (OR2) (in mM: 82.5 NaCl, 2.5 KCl, 1 MgCl2, 1 CaCl2, 1 Na2HPO4, and 5 HEPES, pH 7.5). Whole cell membrane current of single oocytes was measured using a two-microelectrode voltage clamp and recorded with a chart recorder. Both voltage-measuring and current-passing microelectrodes (~1.0 MΩ resistance) were pulled with a vertical Puller (Kopf) and filled with 3 M KCl. The recording chamber was perfused continuously with solution. The TAT-P2X7 peptides (see below) were applied for 30 min, and oocytes were washed by perfusion with OR2 before testing with 500 µM ATP.

The holding potential was −30 mV.

**Small interfering RNA.** J774 cells were treated with 2 µg/1.5 ml small interference RNA (siRNA) corresponding to the mouse Panx1 sequence using 6 µl/1.5 ml oligofermentative reagent (Invitrogen), as previously described (30). After overnight exposure, transfection reagents were removed, and cells were incubated for 30–48 h in DMEM-FBS medium.

**Immunoprecipitation and Western blots.** Confluent cultures of J774 cells untreated and treated with Panx1 siRNA were lysed in Tris-buffered solution containing 1% Triton X-100 (Sigma) and protease inhibitor cocktail (Sigma). Preclearred lysates were incubated for 3 h with anti-P2X7 (Alomone Laboratories), with anti-phosphotyrosine (Upstate, Millipore) or with anti-Panx1 antibodies (see Ref. 30) at 4°C before the addition of agarse-conjugated IgG (UltraLink Immobilized Protein G; Pierce) or IgY (when using anti-Panx1 antibodies; ProSci) beads. After overnight incubation at 4°C, immunocomplexes were washed five to six times with lysis buffer solution, and bound proteins were eluted with 2× Laemmlí buffer containing 100 mM dithiothreitol (DTT). Samples of immunoprecipitated proteins were subjected to electrophoresis using SDS-PAGE minigels (4–20%; Bio-Rad). Proteins were transferred to nitrocellulose membranes (Schleider & Schuell), and, after overnight blocking (2% nonfat dry milk in 1× PBS with 0.05% Tween 20), membrane were blotted using either anti-P2X-R (1:500) or anti-Panx1 (1:2,000) for 2 h at room temperature. After several washes in 1× PBS-Tween 20 solution, membranes were incubated for 2 h with goat-anti-rabbit or rabbit-anti-chicken horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology). Parallel experiments were performed on whole cell lysates of untreated and Panx1 siRNA-treated J774 cells blotted with anti-P2X-R antibodies (1:500) and goat anti-rabbit HRP-conjugated secondary antibodies. Bands were visualized on X-ray films (Kodak) after incubation with the enhanced chemiluminescence reagent (Amersham). Quantification of Panx1 and P2X-R expression levels was performed using NIH image-J software.

**Src phosphorylation.** Confluent cultures of J774 cells plated on 100-mm dishes were exposed for 15 min to BzATP (300 µM) in the absence or presence of the murine P2X-R antagonist KN-62 (1 µM), the TAT-P2X7 peptide (10 µM), and the src tyrosine kinase inhibitor PP2 (10 µM). After 5 min, cells were lysed in Tris-HCI (50 mM; pH 7.2), NaCl (150 mM), NaF (40 mM), EDTA (5 mM), EGTA (5 mM), Nonidet P-40 (NP-40, 1%), Triton X-100 (1%), sodium deoxycolate (0.1%), phenylmethylsulfonyl fluoride (PMSF, 1 mM), and protease inhibitor cocktail (Complete-EDTA-free tablets; Roche). Supernatants (10,000 revolutions/min for 8 s) of whole cell lysates were diluted in 2× Laemmlí buffer containing 100 mM DTT and electrophoresed in 4–20% minigels. Total protein (50 µg) was loaded in each lane. Nitrocellulose membranes were blotted with rabbit anti-Src[pY418] (phosphospecific antibodies (1:500 dilution; Biosource) for 2 h at room temperature, washed, and then incubated with goat anti-rabbit HRP-conjugated antibody (1:2,000; Santa Cruz Biotechnology). After detection of bands, membranes were reprobed using the rabbit anti-pan-src (1:500; Biosource). Quantification of activated Src was performed by measuring the ratio of src[pY418] to pan-Src bands after densitometric analysis using NIH J-image software.

**Competition assays.** Confluent cultures of J774 cells grown in 100-mm dishes were lysed in a buffer solution containing 50 mM Tris-Cl (pH 7.2), 150 mM NaCl, 40 mM NaF, 5 mM EGTA, 1% NP-40, 0.1% sodium deoxycholate, 1 mM Na3VO4, 1% Triton X-100, 1 mM PMSF, and complete protease inhibitor (Roche). Lysates were precleared by incubation with IgG agarse-conjugated beads (Ultra-Link Immobilized Protein G; Pierce) for 120 min at 4°C and brief centrifugation. Precleared lysates were used for competition assays. Typically, 100–200 µl of precleared lysates were incubated with different concentrations (0–300 µM) of the TAT-P2X7-peptide (SLHDSPPPTPGGQGYKRRQQR 451P) for ~15 min at 4°C, before immunoprecipitation assays using anti-P2X7 antibodies (see above).

**Dye uptake.** J774 cells plated on glass-bottomed dishes (MatTek) were bathed for 5 min in a Ca2+-containing or Ca2+-free PBS (pH 7.4) containing the cell-impermeant (629 Da) dye YoPro-1 (5 µM; Invitrogen-Molecular Probes), and basal fluorescence intensity was measured from regions of interest located on cells, as previously described (43). Cells were then exposed to a solution containing 150–300 µM BzATP and 5 µM YoPro, and fluorescence intensity was measured following 5–10 min stimulation. (Higher concentrations of agonist than those in the electrophysiological studies are necessary for the optimal detection of YoPro fluorescence.) After background subtraction, YoPro fluorescence intensity was determined.
Electrophysiology and pharmacology of the P2X7R-Panx1 complex. To elucidate discrepancies in the literature (30, 38, 46) regarding the effects of compounds known to block gap junction channels on Panx1 channels and to verify the molecular identity of the P2X7 receptor permeabilization pore, we used the J774 macrophage cell line to evaluate the pharmacological and electrophysiological properties of the P2X7R-Panx1 complex.

Although there is no specific P2X7R agonist, a pharmacological characteristic of these receptors is their higher sensitivity to BzATP than to ATP (reviewed in Ref. 12). Accordingly, we found that BzATP at lower concentrations induced larger currents in these cells than higher concentrations of ATP when the membrane potential was held at −60 mV. After 5 s BzATP (50 μM) stimulation, a biphasic inward current was recorded with an initial small inward current followed by a larger one (Fig. 1A, top). After ATP (1 mM) application, similar currents were recorded (Fig. 1A, bottom); however, the amplitudes of currents induced by 1 mM ATP were smaller than those induced by a 20-fold lower concentration of BzATP (50 μM), as expected for the P2X7R.

To investigate the possibility that the slowly developing, large-amplitude current recorded following P2X7R stimulation was mediated by Panx1 channels, we applied three compounds that have been shown to affect Panx1 and some gap junction channels (CBX, FFA, and MFQ) and measured current amplitudes evoked by 50 μM BzATP while holding membrane potential at −60 mV. All three compounds greatly reduced agonist-induced currents (Fig. 1, A–C). Incubation (5 min) with 50 μM CBX reduced the amplitudes of BzATP-evoked currents by 87.5% (P < 0.01; n = 6 cells; Fig. 1A, top). The blockade by CBX was reversible; after 2 min washout, the amplitudes of currents induced by BzATP were not significantly different from those measured in the absence of CBX (Fig. 1A). Similarly, CBX (50 μM) reversibly reduced by 83.4% the amplitudes of ATP-induced currents (P < 0.01; n = 6 cells; Fig. 1A, bottom).

Fig. 1. Blockade of P2X7 receptor (P2X7-R)-induced currents by gap junction channel blockers and a P2X7-R antagonist. A: representative current traces induced by 5-s application of 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP, 50 μM; traces on top) and ATP (1 mM; traces on bottom) are shown. Although there is no specific P2X7R agonist, a pharmacological characteristic of these receptors is their higher sensitivity to BzATP than to ATP (reviewed in Ref. 12). Accordingly, we found that BzATP at lower concentrations induced larger currents in these cells than higher concentrations of ATP when the membrane potential was held at −60 mV. After 5 s BzATP (50 μM) stimulation, a biphasic inward current was recorded with an initial small inward current followed by a larger one (Fig. 1A, top). After ATP (1 mM) application, similar currents were recorded (Fig. 1A, bottom); however, the amplitudes of currents induced by 1 mM ATP were smaller than those induced by a 20-fold lower concentration of BzATP (50 μM), as expected for the P2X7R.

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The anti-malarial drug MFQ has been previously reported to block gap junction channels in a connexin-specific manner (14) and to block the uptake of fluorescent molecules in cells expressing the P2X7-R (46). Incubation of J774 cells for 2 min with 1 μM MFQ reduced BzATP-induced current by 78% (Fig. 1C; P < 0.01; n = 5 cells). After 5–6 min MFQ washout, current amplitudes were restored to their original values (Fig. 1C).

The amplitudes of the initial small currents induced by 50 μM BzATP (51.5 ± 3.7 pA; n = 18 cells) were not significantly different (P > 0.5; ANOVA) from those recorded in the presence of CBX (44.3 ± 5.6 pA; n = 5 cells), FFA (53.5 ± 7.0 pA; n = 5 cells), and MFQ (48.2 ± 3.7 pA; n = 6 cells). Thus these results showing that CBX, FFA, and MFQ blocked the slowly developing large-amplitude current but not the fast-developing and smaller-amplitude current suggest that these two currents may be mediated by distinct molecular pathways: the small cation conductance due to opening of the P2X-R and the large conductance provided by Panx1 channels. To verify that the initial conductance insensitive to CBX, FFA, and MFQ was related to ion influx through the ionotropic receptor, we used KN-62, a known P2X7-R antagonist (4). Two minute preincubation of J774 with 1 μM KN-62 completely blocked both components of the BzATP-induced currents (Fig. 1D; n = 4 cells). To further test for the involvement of Panx1 in the late response, we transfected cells with Panx1 siRNA overnight and tested responses 30 h later. Knockdown of Panx1 with siRNA reduced by 40% the second component of BzATP-induced current, without affecting the small cation conductance (control=51.4 ± 3.7, n = 18 cells; siRNA=48.5 ± 6.3 pA, n = 7 cells; P > 0.5, t-test) (Fig. 1E).

Together, these results strongly indicate that the initial conductance insensitive to CBX, FFA, and MFQ corresponds to the opening of P2X-R channels and that the later large-conductance current sensitive to these three compounds corresponds to opening of Panx1 channels. Moreover, we found that activation of P2X-R is necessary for the opening of Panx1 at negative (-60 mV) membrane potentials, given that blockade of P2X-R with KN-62 prevented Panx1 currents (Fig. 1D).

Besides being activated through P2 receptors (30, 31; see Fig. 1), Panx1 channels are voltage gated, opening at positive membrane potentials (9). Further support for the functional presence of Panx1 channels in J774 cells is shown in Fig. 2. As previously reported in J774 macrophages (38), we also found an increase in whole cell currents at voltages above +20 mV, consistent with the presence of Panx1 in this cell line (Fig. 2A).

These outward currents shared a similar pharmacological profile to Panx1 currents when exogenously expressed in Xenopus oocytes (9); specifically, these outward currents were greatly attenuated by 50 μM CBX and by higher concentrations of FFA (0.3 mM). Moreover, we found that the anti-malarial drug MFQ (50–100 nM) but not the P2X7-R antagonist KN-62 (1 μM) greatly attenuated voltage-induced Panx1 outward currents (Fig. 2A). [The remaining currents, not sensitive to CBX, FFA, and MFQ, are unlikely to be tetraethylammonium-sensitive potassium channels given that reversal potential, measured by applying voltage steps after a ramp protocol, was found to be very close to 0 mV (E<sub>rev</sub> = −3.0 mV; N = 3; data not shown]). Further support that Panx1 is responsible for the outward currents sensitive to CBX, FFA, and MFQ was obtained from experiments using Panx1 siRNA. Treatment (48 h) of J774 cells with Panx1 siRNA reduced current amplitude by 35% (Fig. 2A).

Evidence that Panx1 is involved in P2X7-R-mediated membrane permeabilization was obtained by measuring YoPro uptake in J774 cells treated with Panx1 siRNA and with CBX and MFQ (Fig. 2B). Each of these treatments led to >90% decrease in YoPro uptake. That Panx1 siRNA effectively reduced (61.7 ± 0.003%; n = 3 independent experiments) Panx1 expression levels is shown in Fig. 2C. Because we performed immunoprecipitation assays using P2X7-R antibodies to evaluate the levels of Panx1 knockdown, these results also indicate that Panx1 is present in the P2X7-R complex, as previously shown (38). Figure 2D shows representative fluorescence images of YoPro uptake in untreated and Panx1 siRNA-treated J774 cells to illustrate that, besides reducing the amount of dye uptake, knockdown of Panx1 caused a 69.40 ± 0.08% (n = 3 experiments) decrease in the number of cells loaded with the dye in response to 300 μM BzATP.

P2X7-R-Panx1 signaling pathway. To gain insight into pathways that could mediate the opening of Panx1 channels following activation of P2X-R, we considered the possibility that the proline-rich region located at the COOH-terminal domain of the P2X7-R, which has been previously reported to be an important site involved in cell permeabilization (schematic in Fig. 3A; see Refs. 1 and 28), could be involved. To test for that possibility, we designed membrane-permeant TAT-fusion peptides spanning that domain (see MATERIALS AND METHODS) and evaluated effects on membrane permeabilization and on BzATP-induced currents. As shown in Fig. 3B, J774 macrophages treated with 10 μM TAT-P2X7 peptide for 15 min, but not cells treated with heat-inactivated TAT-P2X7 peptide, showed a substantial decrease in the amount of YoPro uptake following 150 μM BzATP.

Electrophysiological recordings performed on J774 cells indicated that the late currents induced by 5 mM ATP were attenuated when cells were exposed to the TAT-P2X7 peptides (Fig. 3C). However, due to the large variability in the amplitudes of these late currents induced in individual cells by high concentrations of ATP in TAT-P2X7 peptide untreated cells, no significant difference was obtained compared with treated cells. Intracellular localization of active and heat-inactivated TAT-P2X7 peptides was confirmed optically using fluorescein isothiocyanate-conjugated peptides [Supplemental Figs. S1 and S2; Supplemental material for this article is available online at the American Journal of Physiology: Cell Physiology website.].

To better control for such variability, which is likely related to differences in P2X7-R and Panx1 expression levels in this macrophage cell line, we used Xenopus oocytes coexpressing P2X7-R and Panx1 to evaluate the effects of TAT-P2X7 peptides. As shown in Fig. 3, D and E, at a ~30-mV holding membrane potential, TAT-P2X7 peptides greatly reduced the amplitudes of ATP-induced inward currents in Xenopus oocytes coexpressing P2X7-R and Panx1 (Fig. 3D; control = 2.65 ± 0.26 μA; TAT-451P peptide = 0.68 ± 0.13 μA; TAT-451L peptide = 1.00 ± 0.18 μA; P < 0.01; n = 3 independent experiments) without affecting currents of oocytes expressing only P2X7-R.
P2X-R (Fig. 3E; control=0.085 ± 0.011 μA and TAT peptides=0.053 ± 0.005 μA; P > 0.05; n = 4 independent experiments). In oocytes expressing exclusively Panx1, TAT-P2X7 peptide did not decrease membrane conductance when voltage was stepped from −30 to +60 mV. Instead, during TAT-peptide application, oocyte membrane conductance increased 1.47 ± 0.13-fold from control levels and to 1.84 ± 0.20-fold after washout (Fig. 3F). At holding membrane potential (−30 mV), inward currents increased 20% from control after TAT-peptide washout (Fig. 3F, inset).

Together, the dye uptake and electrophysiological data suggest that the proline-rich segment, which contains an SH3 domain of the P2X-R COOH-terminus is likely to be involved in the signal transduction pathway that leads to Panx1 activation following receptor activation. Possible scenarios for such action could involve the interaction and/or the activation of an intermediate molecule, most likely a tyrosine kinase. To answer the question of whether Src-tyrosine kinase participates in this process, we performed competition assays in which TAT-P2X7 peptides were incubated at different concentrations with J774 whole cell lysates before immunoprecipitation with P2X7 antibodies followed by Western blots using anti-Src[pY418] antibodies. Our results revealed a dose-dependent reduction of Src[pY418] bound to the P2X7R complex with an increased concentration of TAT-P2X7 peptides (IC50 = 20 μM; Fig. 4A).

To test the hypothesis that a Src tyrosine kinase is an intermediate protein mediating the activation of Panx1 through...
the P2X7R, J774 cells were treated with the Src tyrosine kinase inhibitor PP2 (10 μM) for 5 min to evaluate BzATP-induced Panx1 currents and membrane permeabilization. As shown in Fig. 4, B and C, PP2 attenuated both BzATP-induced currents and YoPro uptake. Further evidence for the participation of Src in the signal transduction pathway was obtained by measuring the amount of tyrosine-phosphorylated Src (active) in J774 cells stimulated with 300 μM BzATP in the absence and presence of 1 μM KN-62, 10 μM TAT-P2X7 peptide and 10 μM PP2. As shown in Fig. 4D, blockade of P2X-R with KN-62 or interference with Src binding and activation significantly reduced BzATP-induced Src phosphorylation. Thus these results support the hypothesis that Src activation following P2X-R stimulation is likely involved in the process leading to the opening of Panx1 channels.

DISCUSSION

The membrane-permeabilizing action of ATP (formation of a transmembrane pathway for flux of moderately sized molecules) first described as mediated by the so-called purinergic P2X receptor (20) was later identified as being due to P2X-R (33, 47). The physical relationship between the P2X7R cation channel and the nonselective pore was until very recently unclear. Several models for pore formation have been proposed to explain the somewhat variable degree of agonist-induced permeabilization among various cell types; these include the recruitment of monomeric channel subunits (12, 13, 48) and the recruitment of a lytic pore (34), which has been recently suggested to be Panx1 (30, 38).

Pannexins are a newly discovered (but phylogenetically old) group of proteins that share no sequence homology with the vertebrate gap junction proteins, connexins, and a low but significant homology with the nonchordate gap junction proteins, the innexins (5, 35, 36, 52). When expressed in Xenopus oocytes, Panx1 and Panx2 have been reported to form gap junction channels to some extent and also to function as gap junction hemichannels that are sensitive to compounds known to block gap junction channels, including CBX and FFA (9, 10). Gap junction channel formation by pannexins in mammalian cells remains to be conclusively demonstrated (16) and is unlikely due to the presence of a glycosylation site in the extracellular loop of this protein (7, 8, 37). However, Panx1 readily forms nonjunctional channels (pannexons) that are voltage sensitive and large conductance (400–500 pS) and have been reported to be modulated by intracellular calcium and by mechanical stretch (3, 31); these large-conductance channels have been proposed to mediate ATP release from...
erythrocytes and mouse taste buds (22, 29) and to underlie dye uptake and ATP release formerly attributed to connexin43 hemichannels (3; reviewed in Ref. 43).

Our previous studies performed on Xenopus oocytes indicated that Panx1 channels are likely to form the permeabilizing pore of the P2X7R death complex (30). With the use of several cell types, including the J774 macrophage cell line, a previous study (38) provided evidence that P2X7R and Panx1 were coimmunoprecipitated; they also showed that knocking down Panx1 expression by siRNA and blocking Panx1 channels using either Panx1 mimetic peptides or the gap junction channel blocker CBX prevented P2X7R-induced uptake of large dye molecules (38). However, in this report (38), none of these treatments affected ATP-induced currents. This is an unexpected finding given that Panx1 forms large-conductance channels (400–500 pS) that are blocked by CBX (3, 9, 30). Thus it would be expected that Panx1 would contribute significantly to the amplitudes of ATP-induced currents.

In agreement with previous work (38), we found that in J774 macrophages, Panx1 is associated with the P2X7R (Fig. 2C) and that activation of Panx1 channels by means of P2X7R induces uptake of YoPro (Fig. 2B). Contrary to previous findings (38), however, but consistent with our electrophysiological recordings, we found that both CBX and MFQ, which are shown to reduce P2X7R inward currents, prevented YoPro uptake (Figs. 1 and 2). Thus these results indicate that uptake of large dye molecules is associated with the opening of large-conductance channels provided by Panx1.

The pharmacological results described in the present report using the J774 macrophage cell line are in agreement with our previous studies (46) showing that several compounds known to block gap junction channels (CBX, MFQ, FFA, heptanol, and octanol) prevent P2X7R-induced influx of dye molecules in astrocytoma cells. However, our studies are substantially different from those reported by Pelegrin and Surprenant (38); these authors described that, of the three compounds (CBX, MFQ, and heptanol) they employed, only CBX prevented P2X7-induced dye uptake in macrophages and astrocytoma cells. Such contradiction is likely related to the fact that these compounds were used (38) at concentrations that are either not effective or nonselective in blocking P2X7R-induced dye up-
take (46), as in the case of heptanol (30-fold lower) and of MFQ (1,000-fold higher), respectively.

Early studies (6) proposed that the gap junction protein connexin43, which is expressed in the J774 cell line (2, 6, 19), was the permeabilization pore induced by ATP. Such possibility, however, was later discarded with the cloning of the P2X7R (47) and the finding that peritoneal macrophages derived from connexin43-null mice still displayed ATP-induced permeabilization (2). The pharmacological distinction between pannexin and connexin channels can be problematic due to the fact that both are blocked by similar compounds, although at slightly different concentration, as is the case of CBX and FFA (9). However, here we found that MFQ is likely to be a more reliable compound to distinguish between Panx1 and connexin43 channels; our results showed that 100 nM is sufficient to block Panx1 channels, whereas total blockade of connexin43 gap junction channels requires 30 μM MFQ (14).

In the present study, we have not used “pannexin mimetic peptides” as a diagnostic tool for channel identification. Although such peptides might be expected to affect channels in a sequence-specific way, it has been shown recently that their inhibitory action is nonspecific. Pannexin mimetic peptides, besides inhibiting Panx1 channels, also inhibited the completely unrelated connexin46 channels with similar efficacy (50).

Very little is known about the signaling mechanisms by which activation of P2X7R leads to opening of Panx1 channels. Although influx of calcium has been proposed as a necessary step leading to activation of the P2X7R permeabilization pore (18), it is unlikely that this cation is the sole messenger given that membrane permeabilization was not found to be prevented by chelating intracellular Ca2+ in macrophages (15) or by removing extracellular Ca2+ from oocytes expressing P2X7R and Panx1 (30).

The P2X7R is unique among members of the ionotropic P2XR family because it has a long COOH terminus (~32 kDa) that provides sites for protein-protein interactions (25, 26). The diversity of molecules found to interact with P2X7R indicates that these receptors are likely to mediate distinct functions. Binding of P2X7R to laminin, actin, actinin, integrins, phosphatidylinositol 4-kinase, and receptor protein phosphatase has been reported previously (25); however, there are no reports on the functional significance of such associations. Previous studies (27, 42, 47) have indicated that deletion of the COOH-terminal domain of the P2X7R prevented membrane permeabilization, suggesting that this domain is important for the signal transduction events leading to membrane permeabilization. Analysis of functional consequences of P2X7R polymorphisms has identified a single amino acid mutation (P451L) in the COOH-terminal domain of the rodent P2X7Rs that affected the ability of these receptors to induce membrane permeabilization in immune cells (1, 28).

Based on these reports, we hypothesized that the P2X7R COOH-terminal tail spanning this point mutation, which is also part of the death signal domain (17), is involved in the interaction between P2X7R and Panx1. Our results using membrane-permeant peptides spanning the proline-rich region of the COOH terminus of the P2X7R indicate that this SH3 domain containing the 451-point mutation is necessary to induce influx of large molecules through Panx1 channels. Moreover, these peptides (a decoy for an SH3 binding protein) are here shown to prevent the association between P2X7R and src tyrosine kinase, suggesting that this kinase is likely involved in the initial steps of the signal transduction pathway that leads to membrane permeabilization. This is in agreement with previous studies indicating that tyrosine kinases play a pivotal role in P2X7R-mediated cellular responses (23, 252; but see 15).

Our results thus support a mechanistic hypothesis whereby an Src tyrosine kinase is involved in the initial steps leading to the activation of Panx1 following P2X7R stimulation. Indeed, results presented here showing that an Src tyrosine kinase inhibitor, PP2, greatly attenuated BzATP-induced current and dye uptake in J774 cells provide support for phosphorylation-mediated activation of Panx1 channels. Such a tyrosine kinase-mediated transduction mechanism could either involve the direct tyrosine phosphorylation of Panx1 or the activation of another molecule. Database search (http://www.cbs.dtu.dk/services/NetPhos/) for a tyrosine residue on the cytoplasmic domain of Panx1 indicated that Y324 is a likely candidate. Evaluation of tyrosine phosphorylation of Panx1 performed on Western blots derived from immunoprecipitates (with anti-phosphotyrosine antibodies) of whole cell lysates of J774 cells that were treated with BzATP in the absence and presence of KN-62, TAT-P2X7 peptide, and PP2 did not reveal that Panx1 is tyrosine phosphorylated (unpublished observations). Although these results strongly suggest that activation of Panx1 channels through the P2X7R involves the phosphorylation of another molecule, the identity of such molecule remains to be determined.

In conclusion, our studies favor the hypothesis that Panx1 is most likely the permeation pore induced by the P2X7R in the J774 macrophage cell line and that a Src tyrosine kinase is one of the molecules that is involved in the transduction of ATP signals into membrane permeabilization.

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